Escalating levels of glyphosate resistance in Iowa populations of *Conyza canadensis* (horseweed): implications for fitness effects

Research Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in Evolution & Ecology in the undergraduate colleges of The Ohio State University

By

Emily Ernst

The Ohio State University
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Project Advisor: Dr. Allison Snow, Department of Evolution, Ecology, & Organismal Biology

Abstract

Glyphosate, sold as RoundUp, is the most commonly used herbicide worldwide and more than 30 weed species have evolved resistance to it. In *Conyza canadensis* (horseweed or marestail), glyphosate resistance was first reported in 2000 in Delaware, while Iowa has only seen resistance since 2011. The goals of this experiment were to 1) test for habitat differences in the level of glyphosate resistance, and 2) test for differences in fitness traits (rosette size and biomass) between susceptible and resistant biotypes in Iowa. I hypothesized that greater levels of glyphosate resistance would be found in agricultural populations than non-agricultural, and that resistance would not be associated with slower growth. Seeds were collected from 28 populations in 2013, with one maternal line (biotype) per population, representing 14 biotypes from soybean fields and 14 biotypes from non-agricultural habitats (e.g., roadsides, parks, etc.). Seedlings were grown in a greenhouse and sprayed with glyphosate after six weeks using three dosages: 1X (= 0.84 kg ae glyphosate/ha), 8X, and 20X, with 0X as a control. With 24 plants in each dosage treatment, per cohort, biotypes with at least 80% survival at each dosage were designated as "resistant" (1X), "highly resistant" (8X), or "extremely resistant" (20X), respectively. I found that 93% of non-agricultural biotypes and one agricultural biotype were susceptible at 1X. Also, 36% of agricultural biotypes were extremely resistant compared to none from non-agricultural habitats, confirming my first hypothesis. Differences in rosette size between susceptible *vs.* resistant biotypes were significant ($p < 0.0001$), resulting in larger resistant plants, and differences in biomass were not significant. My results suggest that glyphosate resistance is likely to persist and may increase in frequency and strength if selection pressures from glyphosate applications continue and if seeds of resistant biotypes disperse into

non-agricultural areas. Worldwide, the spread of glyphosate-resistant weeds is causing growers to use herbicide mixtures that include other modes of action.

Introduction

Resistance

Since their introduction in the 1970s, herbicides have become increasingly relied upon for weed management (Nandula et al. 2005). Combined with this dependency on herbicides, increasing herbicide resistance is commonly found in weeds across agricultural lands and even resistance to multiple herbicides in some weeds (e.g., Walsh et al. 2004, Yu et al. 2007, Legleiter and Bradley 2008, Heap 2015). My study focused on glyphosate resistance. Glyphosate, the main component in RoundUp, is the most common herbicide worldwide, and is used in RoundUp ready, no-till soybean, cotton, and corn production (Baylis 2000, Young 2006). Overuse of herbicides has been one of the main ethical concerns since the creation of herbicides (Radosevich et al. 1992) and is also a cause of decreased herbicide efficiency at normal field rates in weeds (Owen 1997).

Glyphosate (*N*-(phosphonomethyl)glycine) is a broad-spectrum, systemic herbicide whose toxicity inhibits the production of EPSPS, a vital metabolic enzyme in the shikimate pathway (Franz et al. 1997, Herrmann and Weaver 1999). Two types of adaptations for herbicide resistance have been recognized: target site mutations and nontarget site mechanisms (Sammons and Gaines 2014). In *Conyza canadensis*, nontarget site mechanisms, reduced translocation and rapid glyphosate sequestration in vacuoles, are the primary resistance mechanism (Feng et al. 2004, Sammons and Gaines 2014). Vacuolar sequestration is described as an active process induced by glyphosate treatment (Peng et al. 2010). No target site mutations within the EPSPS gene have been found in *C. canadensis* (Nol et al. 2011).

With herbicide resistance, it is expected that there are fitness costs in the absence of the selective pressure due to negative pleiotropic effects (Vila-Aiub et al. 2015). However, many studies show either no difference between resistant and susceptible biotypes of *C. canadenesis*, or even a resistant biotype growing faster than a susceptible biotype (Davis et al. 2009, Shrestha et al. 2010). While Vila-Aiub et al. (2015) point out that comparing populations collected from different locations means that one may be comparing locally adapted traits other than resistance level, a greenhouse study could provide a good foundation for future genetic analyses to properly identify resistance related fitness effects.

Study species

Conyza canadensis, known as horseweed or Canada fleabane, is one of the most common weeds with glyphosate resistance found in no-till soybean fields across North America (Buhler and Owen 1997, Weaver 2001, Tozzi and Acker 2014, Heap 2015). Horseweed is a native North American plant that first gained resistance to glyphosate in 2000 in Delaware while Iowa populations have only been known to be resistant since 2011 (VanGessel 2001, Heap 2015). This species is a winter and summer annual that self-pollinates and can produce >200,000 seeds that are wind dispersed (Mulligan and Findlay 1970, Weaver 2001). Wind dispersal is advantageous for horseweed's ability to spread over vast areas and may aid in the spread of glyphosate resistance (Dauer et al. 2006).

Objectives

The objectives of this study were to test for variation in levels and habitat differences in the extent of glyphosate resistance, and to test for differences in fitness traits (rosette size and

biomass) between susceptible and resistant biotypes in Iowa. I hypothesized that greater levels of glyphosate resistance would be found in agricultural populations than non-agricultural, and that resistance would not be associated with slower growth.

Methods

Seed sources

Dr. Micheal Owen (Iowa State University, Department of Agronomy) and his lab group collected seeds from one plant in each of 32 populations in Iowa in October in 2013. Collected seeds were categorized by the habitat in which they were collected so that variation in resistance could be compared between the two habitats later. Plants that were sampled from soybean fields likely survived glyphosate applications because most growers in these areas cultivate RoundUp Ready soybean (Micheal Owen, pers. comm.). Plants sampled from non-agricultural habitats (e.g., roadsides, parks, etc.) were not likely to experience glyphosate use as a selective pressure. Because horseweed is a highly self-pollinating species (Mulligan and Findlay 1970, Weaver 2001), seeds produced from one plant are assumed to be full siblings and very similar genetically. Here, I refer to progeny from each plant as a maternal line or biotype.

Seeds from each biotype were then shipped to the Ohio State University; I selected 14 biotypes from no-till soybean fields and 14 biotypes that originated from non-agricultural habitats. Biotypes were selected based on germination trials and distance from surrounding biotypes; 5 km was used as the minimum distance from adjacent locations,

and most populations were at least 7 km apart, excluding two agricultural families who were only 2.75 km apart (Fig. 1, Table 1).

Greenhouse experiment

Progeny from each biotype were grown in a greenhouse at Ohio State University and subjected to glyphosate treatments to compare levels of resistance and plant size prior to spraying. For ease of management, I divided the experiment into two cohorts. The first cohort was planted on 25 June 2014 and the second cohort three weeks later. Each cohort included two trays per biotype per treatment, with 6 plants per tray, for a total of 24 plants in each biotype/treatment combination in the whole experiment.

Seeds were grown in a 12.4 cm x 16.8 cm x 5.9 cm tray using Fafard 3B potting soil mix. After 1.5 weeks of growth, trays were thinned to six evenly spaced plants per tray and moved to a greenhouse bench at randomized positions. This was the only time the plants were randomized. The greenhouse was kept at ambient temperatures and all trays were watered using an automatic watering system. The plants received water three times a day for one minute. Supplemental lights were used to simulate a 14-hour day and a 10-hour night. In September 2014, Cohort #2 had to be moved to a different greenhouse where these plants experienced natural day lengths. No fertilizer was added to the trays.

Horseweed plants produce rosettes prior to bolting, and the longest leaf length for each plant was used to measure rosette size at six weeks after germination. Leaf lengths of each plant were recorded using digital Vernier calipers two days before they were sprayed.

Then, the biotypes were exposed to one of four levels of glyphosate: 0X, 1X, 8X, and 20X. These dosages of glyphosate were equivalent to 0.84 kg ae/ha (1X), 6.72 kg ae/ha (8X), and 16.8 kg ae/ha (20X), where 1X was the original recommended field rate for non-resistant weed populations. Using a semi-automated spray chamber, I sprayed the plants with a mixture containing a dosage of glyphosate, 25 mL ammonium sulfate, and 2.5 mL WinField's preference nonionic surfactant. The control of 0X involved no mixture and only water sprayed on the plants. After spraying, the trays were returned to the bench at random positions grouped by treatment level.

Damage and survival scores were recorded at 21 and 42 days after treatment (DAT). Damage was judged visually, based on the percent of the plant that was still alive. Each plant was assigned a score of 1-5, with 1 (0% green) being dead and 5 (100% green) being healthy with no signs of harm from the glyphosate treatment. Living aboveground biomass was also collected 42 DAT by removing any dead plant matter and then cutting the plant where it met the soil. The collected biomass was then placed in a drying oven at ~30^{o}C for at least two weeks. Once the plants were dry, biomass was recorded using an analytical scale.

Data analysis

JMP 11 Pro was the statistical package used for all following computations and analyses. Average leaf length and biomass measurements were first transformed using the $\log_{10}$ for normalizing the data. Averaged leaf lengths and biomass for each tray (N = 6 plants per tray) of resistant and susceptible biotypes were then compared using a nested effects model and Tukey-Kramer tests. The nested effects include resistant level, then families

nested within those levels were considered main effects. Cohort was used as a random variable.

Damage scores were used to determine resistance levels. Biotypes with at least 80% survival at each dosage were designated as "resistant" (1X), "highly resistant" (8X), or "extremely resistant" (20X), respectively with N = 96 plants per family per cohort. Proportions of plants in each resistance category within habitats were compared using a likelihood ratio.

Results

Variation in glyphosate resistance

Of the 28 horseweed biotypes, 14 were susceptible, 6 were resistant, 3 were highly resistant, and 5 were extremely resistant; same resistance level for each family were seen in each cohort (Fig. 1). A higher proportion of susceptible families were found in northern Iowa, while more resistant families were found in the south (Fig. 1). The proportions of biotypes in each resistance category from both habitats were found to be variable with a larger proportion of susceptible biotypes found in non-agricultural habitats (Fig. 2). A likelihood ratio ($\chi^2 = 208.615$) comparing the proportion of biotypes in each category and habitat revealed a significant relationship ($p < 0.001$) between resistance level and habitat. Responses for each cohort shown separately because I found biotypes x cohort interactions in the ANOVA.

Most biotypes that were resistant to glyphosate recovered to be at least 50% of their unsprayed size when sprayed and many were similar to unsprayed plants. In general,

biotypes in the "extremely resistant" category were found to recover from the 1X treatment to their expected final biomass (0X measurements) more so than those in the "resistant" or "highly resistant categories (Fig. 3).

Size of unsprayed biotypes

With possible fitness costs expected with adaptation to resistance, measuring unsprayed biotypes allows for better interpretation of possible effects due to resistance (Vila-Aiub 2015). I used rosette size as a measure of growth prior to spraying. Leaf lengths were averaged for each tray (N = 8 trays per cohort) for every biotype. The means of susceptible and resistant biotypes (resistant, highly resistant, and extremely resistant categories were combined here) were then compared using a nested effects model. Resistant biotypes were found to be significantly larger ($p < 0.0001$), however there were significant interactions of cohort and biotype (both $p < 0.0001$). A Tukey-Kramer test also revealed that two families' average leaf length were significantly ($p < 0.0001$) larger than all other families in Cohort 1 and one family's average leaf length was significantly ($p < 0.0001$) larger in Cohort 2 (Fig. 4, Table 2).

I also used biomass in the 0X treatment as a measure of plant growth. Biomass was averaged for each tray (N = 2 trays per cohort) for every biotype. A nested effects model showed no significant difference between resistant and susceptible biotypes ($p = 0.9945$), however cohort and family effects were found to be significant (both $p < 0.0001$) (Figure 5, Table 3).

Discussion

The variation in glyphosate resistance across habitats indicates that in the four years that it has been witnessed in Iowa, resistance has yet to be fully integrated into the environment outside of agricultural lands. A larger sampling effort may be required to provide a more thorough understanding of the true variation within the state, as susceptible and resistant biotypes can co-occur in the same field. As such, sampling occurred long after initial applications of glyphosate and thus sampling time may have affected the final outcome of these results. Future predictions of resistance between habitats would the blending of non-agricultural populations into greater resistant categories, as is seen in Ohio populations (Beres, unpublished data).

Very high levels of resistance occur in plants from soybean fields in southeastern Iowa. This may be a result of higher use of tillage as a form of weed management combined with herbicide use in the northern part of the state (Micheal Owen, personal communication). Further investigation is required, perhaps through remote sensing techniques, to confirm this relationship.

This study supports the findings reported in Shrestha et al. (2010) where they found faster growth in glyphosate resistant biotypes of *C. canadensis.* They used plant height in a field experiment as a measure of growth and compared heights between one resistant biotype and one susceptible biotype. Another study used dry biomass as an indicator of growth and also determined that the resistant biotype grew larger (Grantz et al. 2008, Alcorta et al. 2011). My study compared 14 resistant and 14 susceptible biotypes and found that, on

average, resistant biotypes initially grew faster than susceptible biotypes at six weeks of growth with resistant biotypes turning out to be 17% larger in rosette size. However, stronger evidence should still be sought after due to significant differences between cohorts even though treatment of each cohort was similar. Further experiments might benefit from using the resistant biotype A8 (see Table 1) as a seed source, if rosette size is heritable and correlated with lifetime fitness. As for biomass, more replications in the experimental design would make these results more reliable because my results were only based on two samples per family per cohort.

This study suggests that glyphosate resistance is likely to persist and may increase in frequency and strength if selection pressures from glyphosate applications continue, and if seeds of resistant biotypes disperse into both agricultural and non-agricultural areas.

Acknowledgments

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Figures & Tables

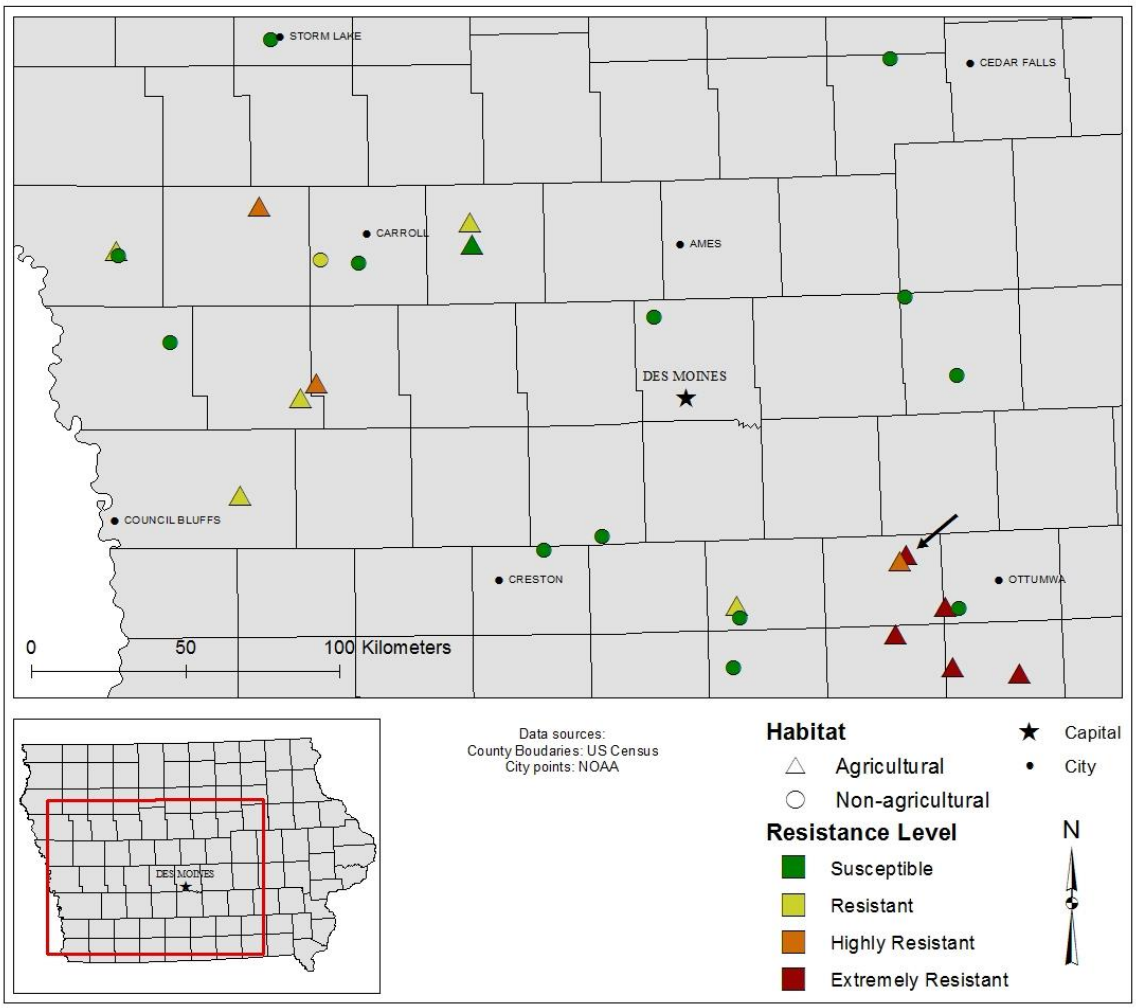

Figure 1: Map showing the location of biotypes with resistance levels and habitats. The arrow points to the biotype that was significantly larger than all others based on a Tukey-Kramer test ($P<0.05$, See Figure 4).

ID	Original ID	Latitude (N)	Longitude (W)	Location	Habitat	Size of population	Field Notes
N1	IANorthNonAg #2	42.63516	-95.23772	North IA	Non-agricultural	100-500	near lake
N2	IANorthNonAg #4	42.54234	-92.77068	North IA	Non-agricultural	<100	road ditch
N3	IANorthNonAg #3	42.01112	-95.84671	North IA	Non-agricultural	<100	road ditch
N4	IASouthNonAg #6	41.99385	-95.04761	South IA	Non-agricultural	<100	road ditch
N5	IASouthNonAg #8	41.98486	-94.89854	South IA	Non-agricultural	<100	road ditch
N6	IASouthNonAg #4	41.8487	-92.7464	South IA	Non-agricultural	<100	road ditch
N7	IASouthNonAg #13	41.81041	-93.73504	South IA	Non-agricultural	>500	state park road ditch
N8	IASouthNonAg #7	41.75866	-95.64418	South IA	Non-agricultural	<100	road ditch
N9	IASouthNonAg #5	41.61626	-92.55224	South IA	Non-agricultural	<100	road ditch
N10	IASouthNonAg #3	41.18038	-93.95865	South IA	Non-agricultural	<100	road ditch
N11	IASouthNonAg #1	41.14264	-94.18765	South IA	Non-agricultural	<100	road ditch
N12	IASouthNonAg #11	40.94123	-92.57894	South IA	Non-agricultural	<100	CRP
N13	IASouthNonAg #12	40.93414	-93.42978	South IA	Non-agricultural	100-500	rd ditch/CRP
N14	IASouthNonAg #10	40.7883	-93.4617	South IA	Non-agricultural	100-500	CRP
A1	IANorthSoyNT #6	42.15325	-95.28921	North IA	Agricultural	>500	
A2	IANorthSoyNT #4	42.09833	-94.4556	North IA	Agricultural	>500	
A3	IANorthSoyNT #7	42.03466	-94.44962	North IA	Agricultural	<100	
A4	IANorthSoyNT #8	42.02406	-95.85538	North IA	Agricultural	<100	
A5	IASouthSoyNT #13	41.63873	-95.06865	South IA	Agricultural	>500	
A6	IASouthSoyNT #14	41.59382	-95.13465	South IA	Agricultural	>500	
A7	IASouthSoyNT #11	41.31396	-95.37312	South IA	Agricultural	100-500	
A8	IASouthSoyNT #25	41.10143	-92.77837	South IA	Agricultural	>500	
A9	IASouthSoyNT #22	41.08499	-92.80298	South IA	Agricultural	>500	glyphosate history
A10	IASouthSoyNT #26	40.96932	-93.44200	South IA	Agricultural	<100	
A11	IASouthSoyNT #18	40.94944	-92.62961	South IA	Agricultural	>500	
A12	IASouthSoyNT #24	40.87429	-92.82976	South IA	Agricultural	>500	
A13	IASouthSoyNT #23	40.77357	-92.60986	South IA	Agricultural	>500	
A14	IASouthSoyNT #16	40.74763	-92.35128	South IA	Agricultural	>500	

Table 1: A table showing the locations of all collected biotypes.

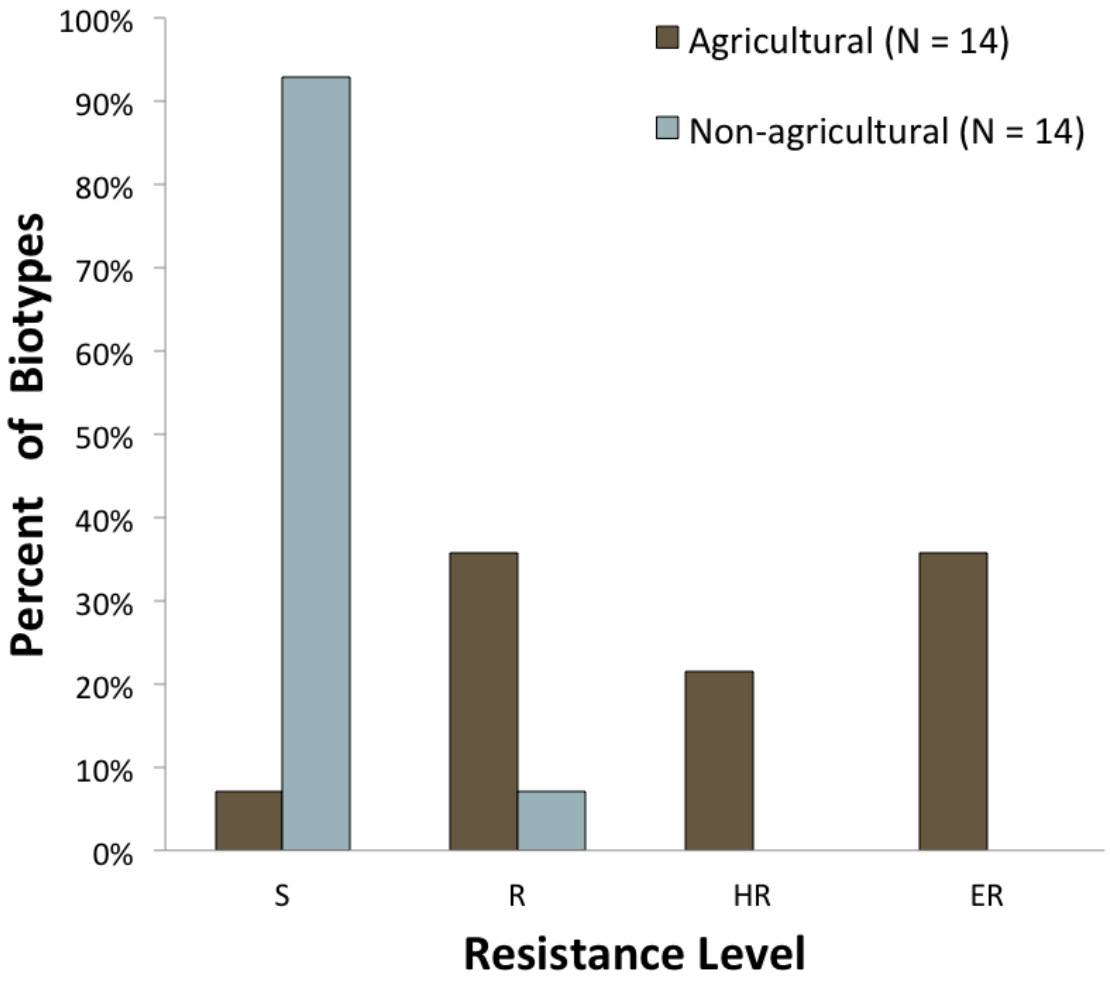

Figure 2: Percent of biotypes grouped by habitat and resistance categories (S = susceptible, R = resistant, HR = highly resistant, ER = extremely resistant).

Effects Test - rosette size

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Resistance level	1	1	2.703479	54.906	<.0001
Biotype[Resistance level]	26	26	54.26435	42.3875	<.0001
Cohort	1	1	1.056631	21.4595	<.0001
Cohort*Resistance level	1	1	0.159066	3.2305	0.0731
Biotype*Cohort[Resistance level]	26	26	14.321789	11.1872	<.0001

Table 2: Results of ANOVA effects test in JMP Pro 11 are depicted above. Resistance level indicates a resistant or susceptible biotype, brackets are used to indicate nested effects, and asterisks show crossed effects.

Effects Test - 0X Biomass

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Resistance level	1	1	3.55E-06	0	0.9945
Cohort	1	1	17.449934	232.8987	<.0001
Resistance level *Cohort	1	1	0.240491	3.2098	0.0786
Biotype[Resistance level]	26	26	24.904073	12.7841	<.0001
Biotype*Cohort[Resistance level]	26	26	11.678272	5.9949	<.0001

Table 3: Results of ANOVA effects test in JMP Pro 11 are depicted above. Resistance level indicates a resistant or susceptible biotype, brackets are used to indicate nested effects, and asterisks show crossed effects.

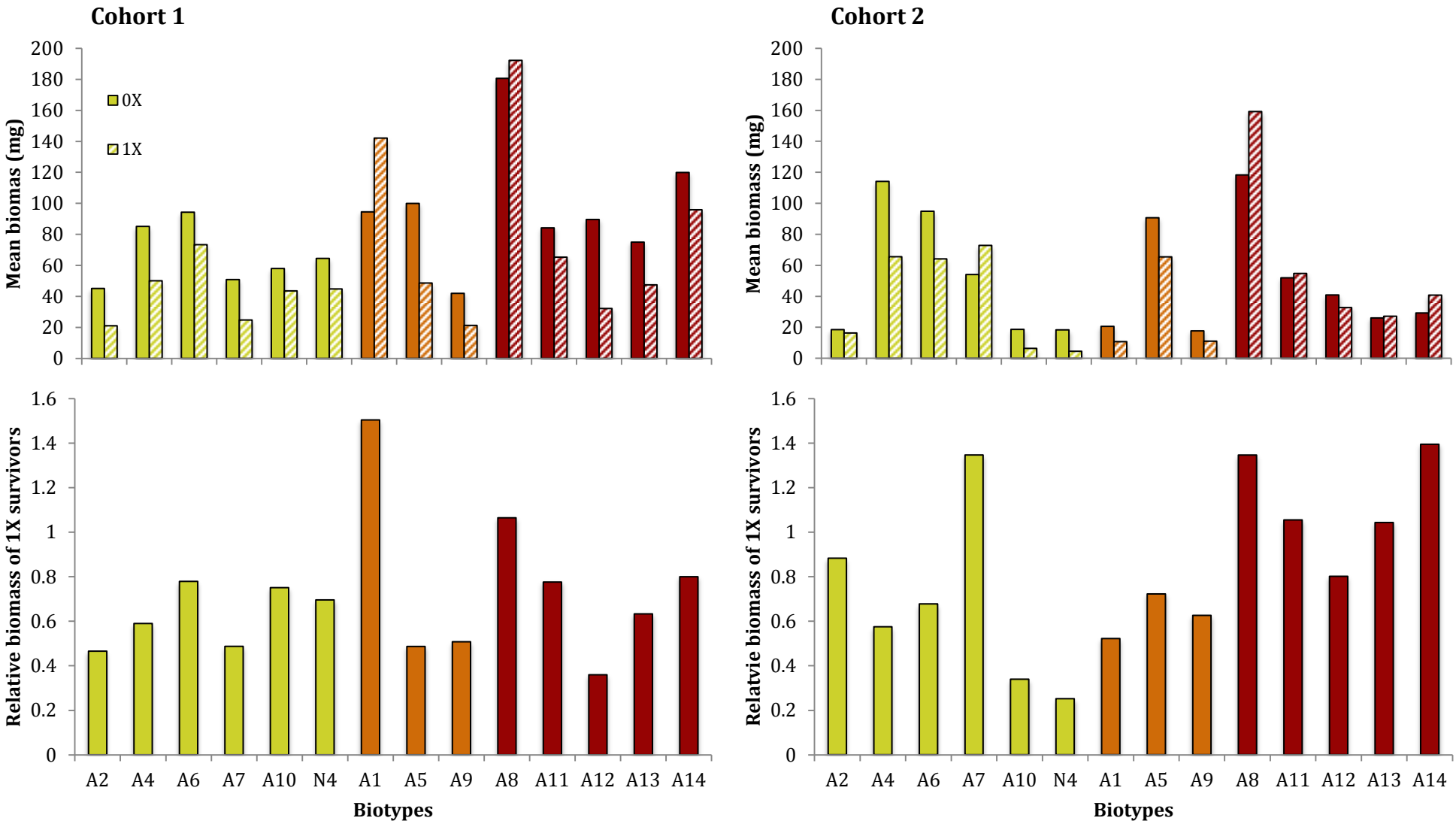
Cohort 1
Mean biomas (mg)
0X
1X
Relative biomass of 1X survivors
A2 A4 A6 A7 A10 N4 A1 A5 A9 A8 A11 A12 A13 A14
Biotypes
Cohort 2
Mean biomass (mg)
Relatvie biomass of 1X survivors
A2 A4 A6 A7 A10 N4 A1 A5 A9 A8 A11 A12 A13 A14
Biotypes

Figure 3: Mean biomass per plant 42 DAT for resistant biotypes in treatments 0X and 1X (N = 2 trays per cohort) with resistance level indicated by color ("resistant" = yellow, "highly resistant" = orange, and "extremely resistant" = red). The top graph shows mean biomass across both treatments while the bottom graph shows the relative biomass, indicating the mean biomass at 0X divided by the mean biomass at 1X.

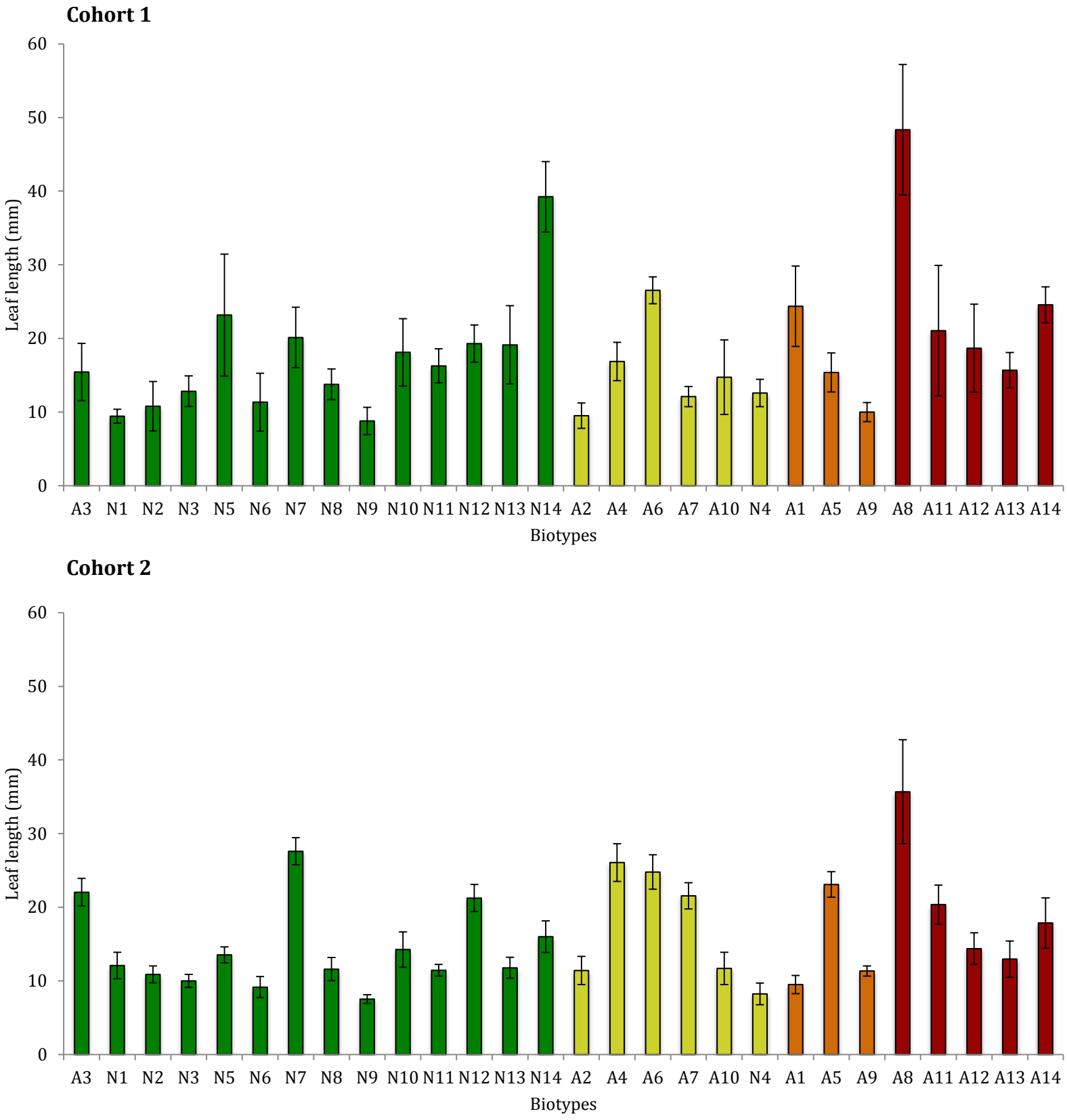

Figure 4: Mean leaf lengths of each biotype after 6 weeks prior to spraying with resistance level indicated by color ("susceptible" = green, "resistant" = yellow, "highly resistant" = orange, and "extremely resistant" = red). 95% confidence intervals are shown. N = 8 trays per biotype, per cohort (data for each tray represents the average of 6 plants).

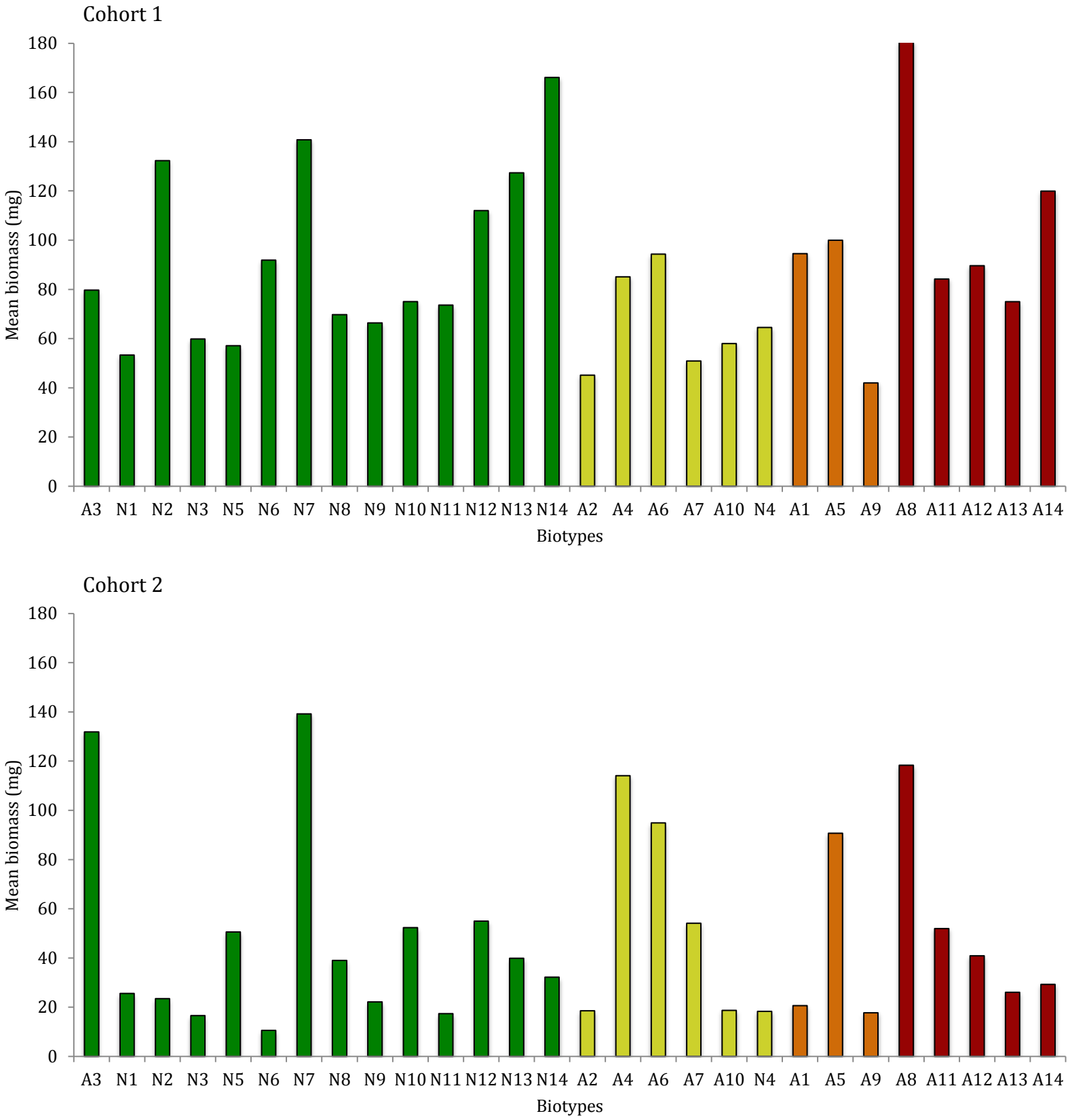

Figure 5: Mean biomass of biotypes in the 0X treatment (N = 2 trays per biotype per cohort) after 13 weeks with resistance level indicated by color ("susceptible" = green, "resistant" = yellow, "highly resistant" = orange, and "extremely resistant" = red).

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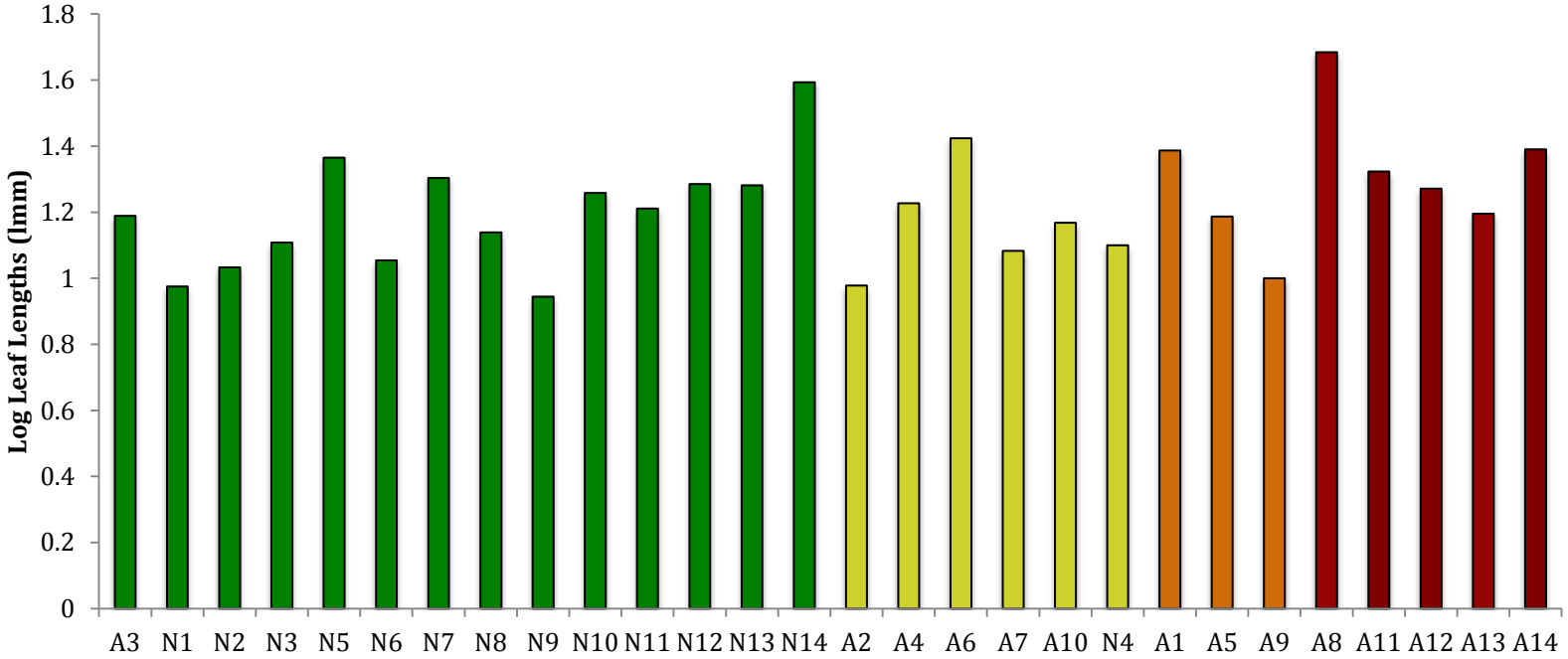
Appendix

Cohort 1



Cohort 2

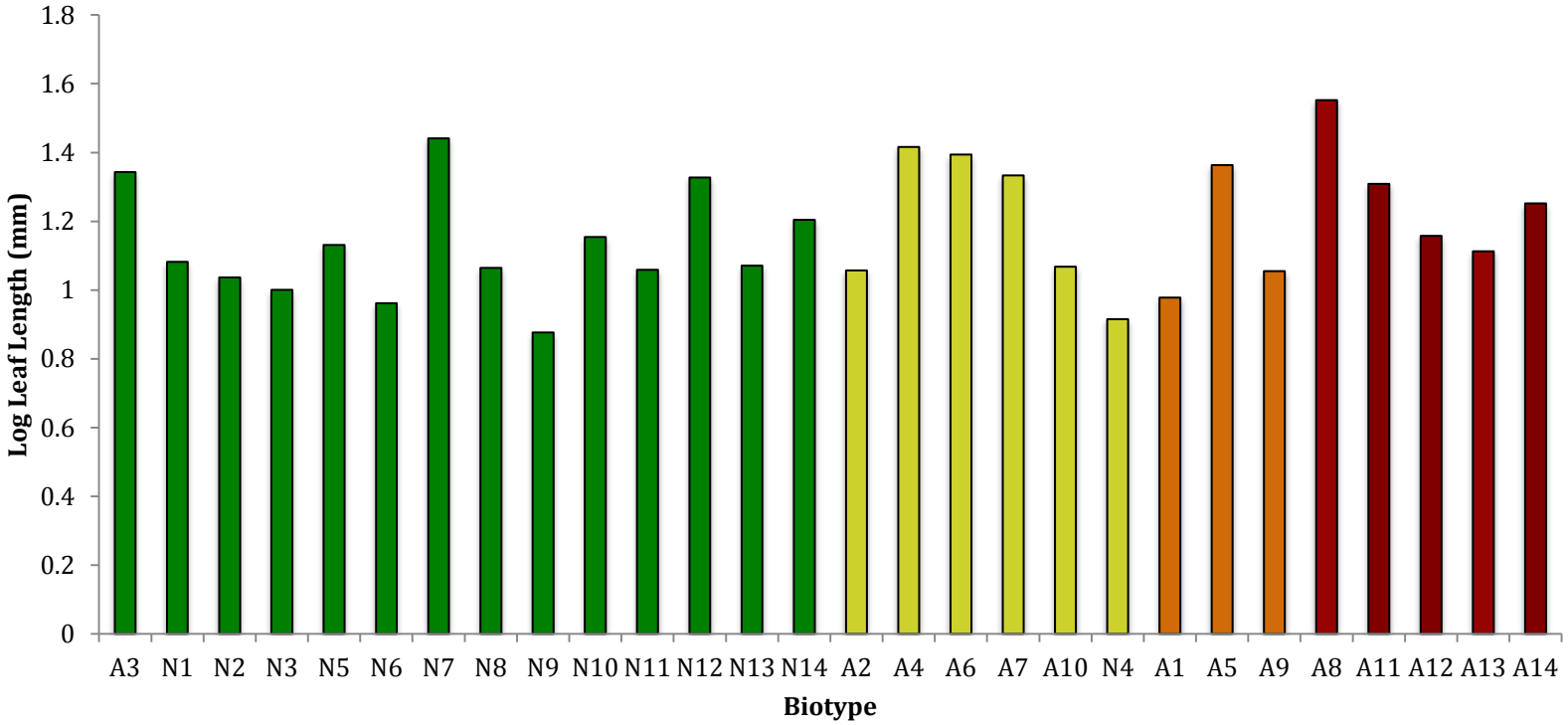
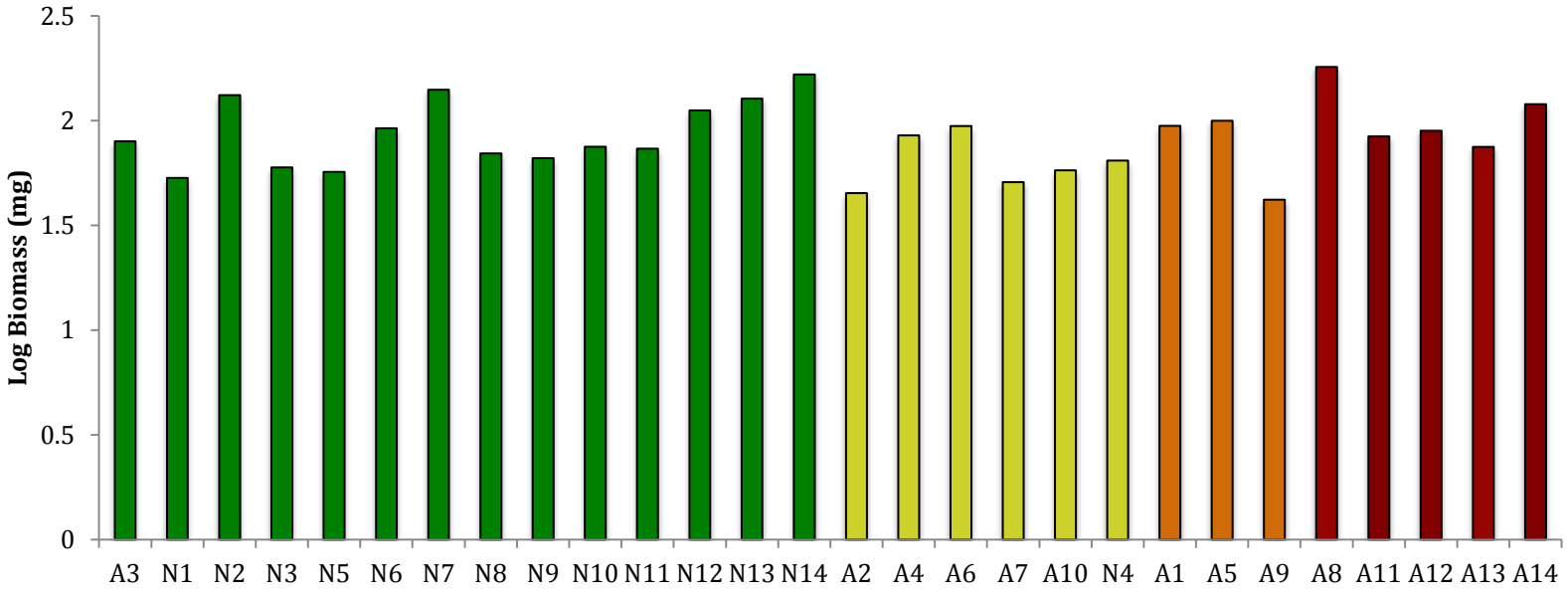


Figure 1: Log mean leaf lengths of each biotype after 6 weeks prior to spraying with resistance level indicated by color ("susceptible" = green, "resistant" = yellow, "highly resistant" = orange, and "extremely resistant" = red). N = 8 trays per biotype, per cohort (data for each tray represents the average of 6 plants).

Cohort 1



Cohort 2

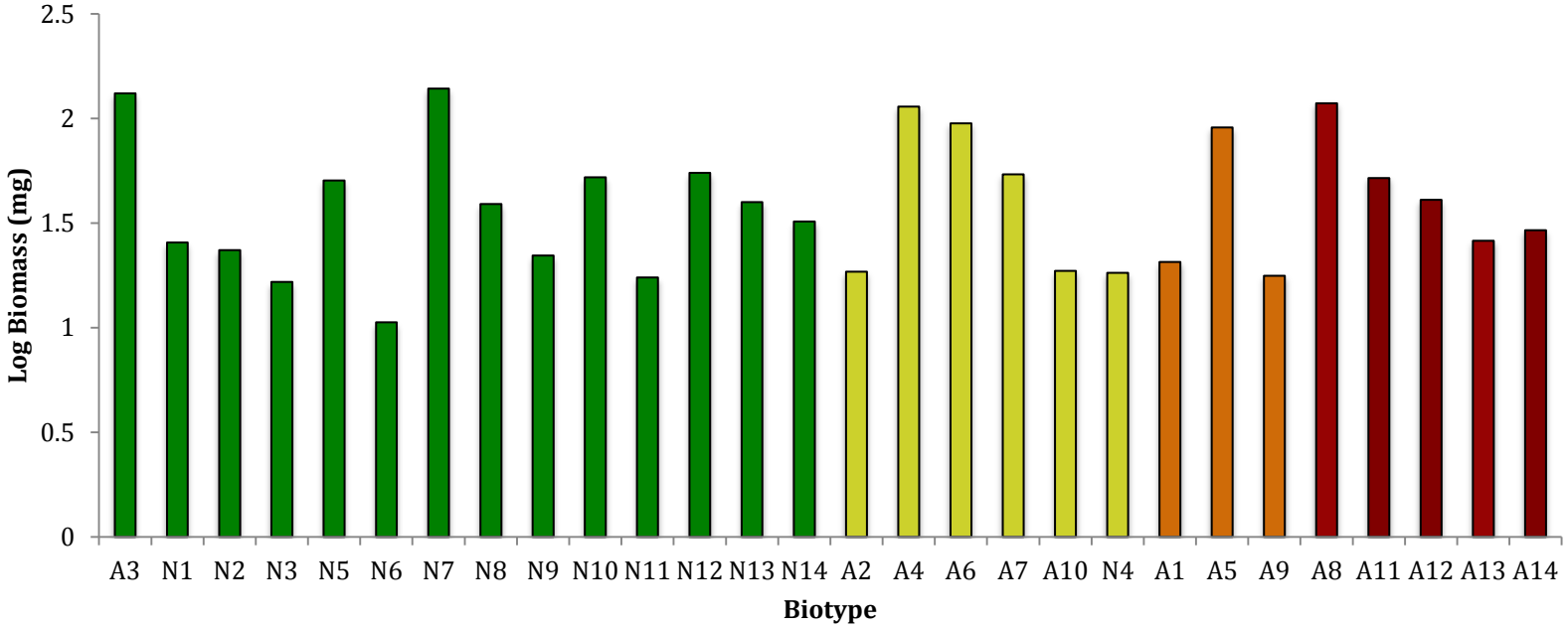


Figure 2: Mean biomass of biotypes in the 0X treatment (N = 2 trays per biotype per cohort) after 13 weeks with resistance level indicated by color ("susceptible" = green, "resistant" = yellow, "highly resistant" = orange, and "extremely resistant" = red).